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(21) International Application Number: PCT/US99/13338 (22) International Filing Date: 11 June 1999 (11.06.1999) (30) Priority Data: 09/119,804 21 July 1998 (21.07.1998) US (60) Parent Application or Grant CHILDREN'S MEDICAL CENTER CORPORATION [/]; O. FOLKMAN, Judah [/]; O. LIN, Jie [/]; O. RESNICK, David, S. ; O.		Published
(54) Title: METHODS AND COMPOSITIONS FOR INHIBITION OF ANGIOGENESIS. (54) Titre: METHODES ET COMPOSITIONS DESTINEES A INHIBER L'ANGIOGENESE (57) Abstract <p>We have now discovered a novel class of antiangiogenic proteins and peptides. Some of the proteins are more potent antiangiogenic agents than presently known proteins such as angiostatin and endostatin. The proteins that can be antiangiogenic agents include those of SEQ ID NOS:1, 2 and 3. We have also discovered pharmaceutical compositions containing an angiogenic inhibitory polypeptide or nucleic acids encoding such a polypeptide, in therapeutically effective amounts that are capable of inhibiting endothelial cell proliferation, and their methods of use.</p> (57) Abrégé <p>La présente invention concerne la découverte d'une nouvelle classe de protéines et de peptides anti-angiogéniques. Certaines de ces protéines constituent des agents anti-angiogéniques plus puissants que les protéines connues à ce jour telles que l'angiostatine ou l'endostatine. Les protéines pouvant agir comme anti-angiogéniques sont celles de SEQ ID NOS:1, 2 et 3. L'invention porte également sur des compositions pharmaceutiques renfermant un polypeptide inhibiteur de l'angiogenèse ou des acides nucléiques codant pour ce polypeptide qui, en doses thérapeutiquement efficaces, sont capables d'inhiber la prolifération de cellules endothéliales. Sont également décrites les méthodes d'utilisation de ces compositions.</p>		

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(21) International Application Number: PCT/US99/13338 (22) International Filing Date: 11 June 1999 (11.06.99) (30) Priority Data: 09/119,804 21 July 1998 (21.07.98) US (71) Applicant: CHILDREN'S MEDICAL CENTER CORPORATION [US/US]; 320 Longwood Avenue, Boston, MA 02115 (US). (72) Inventors: FOLKMAN, Judah; 18 Chatham Circle, Brookline, MA 02146 (US). LIN, Jie; Apartment 3, 40 Newburg Street, Roslindale, MA 02131 (US). (74) Agents: RESNICK, David, S. et al.; Peabody & Brown, 101 Federal Street, Boston, MA 02110 (US).			(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published With international search report.
(54) Title: METHODS AND COMPOSITIONS FOR INHIBITION OF ANGIOGENESIS			
(57) Abstract <p>We have now discovered a novel class of antiangiogenic proteins and peptides. Some of the proteins are more potent antiangiogenic agents than presently known proteins such as angiostatin and endostatin. The proteins that can be antiangiogenic agents include those of SEQ ID NOS:1, 2 and 3. We have also discovered pharmaceutical compositions containing an angiogenic inhibitory polypeptide or nucleic acids encoding such a polypeptide, in therapeutically effective amounts that are capable of inhibiting endothelial cell proliferation, and their methods of use.</p>			

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Description

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METHODS AND COMPOSITIONS FOR INHIBITION OF ANGIOGENESIS

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BACKGROUND OF THE INVENTION

1. Field of the Invention

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The present invention provides for a novel pharmaceutical composition, and method of use thereof for treatment of diseases or disorders involving abnormal angiogenesis.

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2. Background

Blood vessels are the means by which oxygen and nutrients are supplied to living tissues and waste products are removed from living tissue. Angiogenesis refers to the process by which new blood vessels are formed.

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10 See, for example, the review by Folkman and Shing, *J. Biol. Chem.* 267 (16), 10931-10934 (1992). Thus, where appropriate, angiogenesis is a critical biological process. It is essential in reproduction, development and wound repair. However, inappropriate angiogenesis can have severe negative consequences. For example, it is only after many solid tumors are
30 vascularized as a result of angiogenesis that the tumors have a sufficient supply of oxygen and nutrients that permit it to grow rapidly and metastasize. Because maintaining the rate of angiogenesis in its proper equilibrium is so critical to a range of functions, it must be carefully regulated in order to maintain health. The angiogenesis process is believed
35 to begin with the degradation of the basement membrane by proteases secreted from endothelial cells (EC) activated by mitogens such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). The cells migrate and proliferate, leading to the formation of solid
40 endothelial cell sprouts into the stromal space, then, vascular loops are
45 formed and capillary tubes develop with formation of tight junctions and deposition of new basement membrane.

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In adults, the proliferation rate of endothelial cells is typically low compared to other cell types in the body. The turnover time of these cells

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5 can exceed one thousand days. Physiological exceptions in which
angiogenesis results in rapid proliferation typically occurs under tight
regulation, such as found in the female reproduction system and during
wound healing.

10 5 The rate of angiogenesis involves a change in the local equilibrium
between positive and negative regulators of the growth of microvessels. The
therapeutic implications of angiogenic growth factors were first described by
15 Folkman and colleagues over two decades ago (Folkman, *N. Engl. J. Med.*,
285:1182-1186 (1971)). Abnormal angiogenesis occurs when the body loses
at least some control of angiogenesis, resulting in either excessive or
insufficient blood vessel growth. For instance, conditions such as ulcers,
20 strokes, and heart attacks may result from the absence of angiogenesis
normally required for natural healing. In contrast, excessive blood vessel
proliferation can result in tumor growth, tumor spread, blindness, psoriasis
and rheumatoid arthritis.

25 Thus, there are instances where a greater degree of angiogenesis is
desirable-- increasing blood circulation, wound healing, and ulcer healing.
30 For example, recent investigations have established the feasibility of using
recombinant angiogenic growth factors, such as fibroblast growth factor
(FGF) family (Yanagisawa-Miwa, et al., *Science*, 257:1401-1403 (1992) and
Baffour, et al., *J Vasc Surg*, 16:181-91 (1992)), endothelial cell growth factor
(ECGF)(Pu, et al., *J Surg Res*, 54:575-83 (1993)), and more recently,
35 vascular endothelial growth factor (VEGF) to expedite and/or augment
collateral artery development in animal models of myocardial and hindlimb
ischemia (Takeshita, et al., *Circulation*, 90:228-234 (1994) and Takeshita, et
al., *J Clin Invest*, 93:662-70 (1994)).

40 30 Conversely, there are instances, where inhibition of angiogenesis is
desirable. For example, many diseases are driven by persistent unregulated
angiogenesis, also sometimes referred to as "neovascularization." In
45 arthritis, new capillary blood vessels invade the joint and destroy cartilage.
In diabetes, new capillaries invade the vitreous, bleed, and cause blindness.
50 35 Ocular neovascularization is the most common cause of blindness. Tumor
growth and metastasis are angiogenesis-dependent. A tumor must

continuously stimulate the growth of new capillary blood vessels for the tumor itself to grow.

The current treatment of these diseases is inadequate. Agents which prevent continued angiogenesis, e.g. drugs (TNP-470), monoclonal antibodies, antisense nucleic acids and proteins (angiostatin and endostatin) are currently being tested. See, Battegay, *J. Mol. Med.*, 73, 333-346 (1995); Hanahan et al., *Cell*, 86, 353-364 (1996); Folkman, *N. Engl. J. Med.*, 333, 1757-1763 (1995). Although preliminary results with the antiangiogenic proteins are promising, they are relatively large in size and their difficult to use and produce. Moreover, proteins are subject to enzymatic degradation. Thus, new agents that inhibit angiogenesis are needed. New antiangiogenic proteins or peptides that show improvement in size, ease of production, stability and/or potency would be desirable.

SUMMARY OF THE INVENTION

We have now discovered a novel class of antiangiogenic proteins and peptides. Some of the proteins are more potent antiangiogenic agents than presently known proteins such as angiostatin and endostatin. The proteins that can be antiangiogenic agents include those of SEQ ID NOS:1, 2 and 3. We have also discovered pharmaceutical compositions containing an angiogenic inhibitory polypeptide or nucleic acids encoding such a polypeptide, in therapeutically effective amounts that are capable of inhibiting endothelial cell proliferation, and their methods of use.

As used herein an "angiogenic inhibitory polypeptide" refers to a polypeptide having at least one of the following domains: (a) IGF (insulin-like growth factor) binding domain (consensus sequence: GCGCCxxC); (b) vWFC (von Willebrand factor type C repeat, Mancuso et al., *J. Biol. Chem.* 264:19514-19527 (1989)); (c) TSP-1 (Thrombospondin type 1 domain, consensus sequence: WSxCScCG); and (d) CTCK-2 (C-terminal cysteine knot profile, Bork P., *FEBS* 327:125-130(1993)), wherein the polypeptide is an inhibitor of bFGF-stimulated bovine endothelial cell proliferation. That inhibition can be determined by known means such as by using the assay of Folkman et al. (*Natl. Aca. Sci. Proc. USA* 76: 5217-5221, (1979)). Preferably, the protein having at least one of those domains displays those greater than 80%

homology with a protein selected from the group consisting of SEQ ID NO:1 (bovine orthologue for the human connective tissue growth factor, Example 1), SEQ ID NO:2 (human connective tissue growth factor, Bradham et al., *J. Cell Biol.* 114: 1285-1294 (1991)) and SEQ ID NO:3 (*fisp*-12, Ryseck et al., *Cell Growth Differ.* 2:225-233 (1991)).

In another embodiment, the angiogenic inhibitory polypeptide has at least two of the above listed domains, more preferably three and most preferably all four domains.

Angiogenic inhibitory polypeptides are preferably members of a family of growth regulators referred to as the CCN family. *See, e.g.,* review by Bork P., *FEBS* 327:125-130(1993). More preferably, the angiogenic inhibitory polypeptides are mammalian connective tissue growth factors.

In a further embodiment, the invention encompasses polypeptides which have at least about 80% identity compared to a mammalian connective tissue growth factor selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3 and, more preferably, at least about 85% identity. In more preferred embodiment, this identity is greater than 90%. In a still more preferred embodiment, this identity is greater than 95%.

The invention further relates to treatment of neovascular disorders by administration of a pharmaceutical composition comprising an angiogenic inhibitory polypeptide of the invention or nucleic acid encoding such a polypeptide, and a pharmaceutically acceptable carrier. Such angiogenic inhibitory polypeptides include the mammalian connective tissue growth factor of SEQ ID NO: 1, 2 or 3, and fragments and analogs thereof.

In one embodiment, a pharmaceutical composition of the invention is administered to treat a cancerous condition, or to prevent progression from the pre-neoplastic or pre-malignant state into a neoplastic or a malignant state. In other specific embodiments, a pharmaceutical composition of the invention is administered to treat ocular disorders associated with neovascularization.

Other aspects of the invention are disclosed *infra*.

DETAILED DESCRIPTION OF THE INVENTION

We have discovered that the proteins of SEQ ID NOS:1, 2 and 3 display antiangiogenic activity. For example, the protein of SEQ ID NO:1 is more potent than angiostatin as determined by a known assay such as the assay of Folkman et al. (*Proc. Natl. Aca. Sci. USA* 76: 5217-5221, (1979)).

The present invention also relates to therapeutic methods and compositions using "angiogenic inhibitory polypeptides," that have the following properties:

1. have at least one of the following domains: IGF (insulin-like growth factor) binding domain (consens sequence: GCGCCxxC), vWFC (von Willebrand factor type C repeat, Mancuso et al., *J. Biol. Chem.* 264:19514-19527 (1989)), TSP-1 (Thrombospondin type 1 domain, consens sequence: WSxCScCG) and CTCK-2 (C-terminal cysteine knot profile, Bork P., *FEBS* 327:125-130(1993));
2. inhibit bFGF-stimulated bovine endothelial cell proliferation using a known assay; and
3. the peptide segment has greater than 80% homology with the corresponding segment of a protein selected from the group consisting of SEQ ID NO:1 (bovine orthologue for the human connective tissue growth factor, Example 1), SEQ ID NO:2 (human connective tissue growth factor, Bradham et al., *J. Cell. Biol.* 114: 1285-1294 (1991)) and SEQ ID NO:3 (*fisp-12*, Ryseck et al., *Cell Growth Differ.* 2:225-233 (1991)).

Homology is determined using the BLAST program provided by GenBank at the National Library of Medicine. GenBank can be accessed via the Internet at www.ncbi.nlm.gov/.

In a preferred embodiment of the invention, the angiogenesis inhibitory polypeptide is a peptide consisting of at least a fragment of SEQ ID NO: 1, 2 or 3, which is effective to inhibit endothelial cell proliferation using the above assay of Folkman et al.

5 In the above Folkman assay the protein of SEQ ID NO:1 shows a
greater potency than angiostatin and endostatin. One can readily determine
relative antiangiogenic activity by using the activity of a known
10 antiangiogenic compound and comparing the angiogenic inhibition of the
5 proteins and polypeptides of the present invention.

15 In another embodiment, the invention encompasses peptides which
are homologous to bovine connective tissue growth factor (A1) (SEQ ID NO:1)
or fragments thereof. In one embodiment, the amino acid sequence of the
10 peptide has at least 80% identity compared to the fragment of bovine
connective tissue growth factor from which it is derived (the "prototype
fragment"). In another embodiment, this identity is greater than 85%. In a
20 more preferred embodiment, this identity is greater than 90%. In a most
preferred embodiment, the amino acid sequence of the peptide has at least
15 95% identity with the prototype fragment. Fragments can be at least 10
amino acids, and in preferred embodiments at least 50, 75, 100, 120, and
25 200 amino acids, respectively.

30 In another embodiment, the invention encompasses peptides which
are homologous to human connective tissue growth factor (SEQ ID NO:2) or
fragments thereof. In one embodiment, the amino acid sequence of the
peptide has at least 80% identity with the prototype human connective
35 tissue growth factor. In another embodiment, this identity is greater than
85%. In a more preferred embodiment, this identity is greater than 90%. In a
25 most preferred embodiment, the amino acid sequence of the peptide has at
least 95% identity with the prototype fragment. Fragments can be at least
10 amino acids, and in preferred 25 embodiments at least 50, 75, 100, 120,
40 and 200 amino acids, respectively.

30 In another embodiment, the invention encompasses peptides, which
are homologous to *fisp-12* (SEQ ID NO:3), the mouse orthologue of human
45 connective tissue growth factor, or fragments thereof. In one embodiment,
the amino acid sequence of the peptide has at least 80% identity with the
prototype *fisp-12*. In another embodiment, this identity is greater than 85%.
35 In a more preferred embodiment, this identity is greater than 90%. In a most
preferred embodiment, the amino acid sequence of the peptide has at least

5 95% identity with the prototype fragment. Fragments can be at least 10³ amino acids, and in preferred embodiments at least 50, 75, 100, 120 and 200 amino acids in length, respectively.

10 5 In other specific embodiments, the angiogenic inhibitory polypeptides of the invention are human connective tissue growth factor isoforms from other mammalian species, e.g., rabbit, rat, ovine and porcine.

15 Angiogenic inhibitory polypeptides of the invention can be combined
20 with a therapeutically effective amount of another molecule which negatively regulates angiogenesis which may be, but is not limited to, platelet factor 4, thrombospondin-1, tissue inhibitors of metalloproteases (TIMP1 and TIMP2)
25 prolactin (16-Kd fragment), angiostatin (38-Kd fragment of plasminogen), endostatin, bFGF soluble receptor, transforming growth factor β , interferon alfa, and placental proliferin-related protein.

25 An angiogenic inhibitory polypeptide of the invention may also be combined with chemotherapeutic agents.

30 20 Connective tissue growth factor protein analogs, can be made by altering the protein sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. These include, but are not limited to, connective tissue growth factor protein analogs, fragments, or
35 analogs containing, as a primary amino acid sequence, all or part of the amino acid sequence of an connective tissue growth factor protein analogs
25 including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the
40 sequence can be substituted by another amino acid of a similar polarity, which acts as a functional equivalent, resulting in a silent alteration.
30 Substitutes for an amino acid within the sequence may be selected from other members as of the class to which the amino acid belongs. For
45 example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The
35 polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino

acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

One embodiment of the invention provides for molecules consisting of or comprising a fragment of at least 10 (continuous) amino acids of a connective tissue growth factor protein that is capable of inhibiting endothelial cell proliferation. In other embodiments, this molecule consists of at least 20 or 50 amino acids of the connective tissue growth factor protein. In specific embodiments, such molecules consist of or comprise fragments of a connective tissue growth factor protein of at least 75, 120 or 200 amino acids.

In a preferred embodiment, the protein is a mammalian connective tissue growth factor protein. In alternative embodiments, it is a human, bovine or murine connective tissue growth factor protein. The connective tissue growth factor proteins, fragments and analogs of the invention can be derived from tissue or produced by various methods known in the art. The manipulations, which result in their production, can occur at the gene or protein level. For example, a cloned gene sequence coding for connective tissue growth factor proteins can be modified by any of numerous strategies known in the art. Sambrook et al., 1990, *Molecular Cloning, A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of the gene encoding a derivative or analog, care should be taken to ensure that the modified gene remains within the same translational reading frame as the troponin subunit gene, uninterrupted by translational stop signals, in the gene region where the desired troponin activity is encoded.

The connective tissue growth factors are preferably produced by recombinant methods. See the procedures disclosed in Example 1, which follows. A wide variety of molecular and biochemical methods are available for generating and expressing the polypeptides of the present invention; see e.g. the procedures disclosed in *Molecular Cloning, A Laboratory Manual* (2nd Ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor), *Current Protocols*

5 in *Molecular Biology* (Eds. Ausubel, Brent, Kingston, More, Feidman, Smith
and Stuhl, Greene Publ. Assoc., Wiley-Interscience, NY, N.Y. 1992) or other
procedures that are otherwise known in the art. For example, the
10 polypeptides of the invention may be obtained by chemical synthesis,
5 expression in bacteria such as *E. coli* and eukaryotes such as yeast,
baculovirus, or mammalian cell-based expression systems, etc., depending
on the size, nature and quantity of the polypeptide.

15 The term "isolated" means that the polypeptide is removed from its
10 original environment. For example, a naturally-occurring polynucleotides or
polypeptides present in a living animal is not isolated, but the same
polynucleotides or DNA or polypeptides, separated from some or all of the
20 coexisting materials in the natural system, is isolated. Such polynucleotides
could be part of a vector and/or such polynucleotides or polypeptides could
15 be part of a composition, and still be isolated in that such vector or
composition is not part of its natural environment.

25 Where it is desired to express a polypeptide of the invention any
suitable system can be used. The general nature of suitable vectors,
30 expression vectors and constructions therefor will be apparent to those
skilled in the art.

35 Suitable expression vectors may be based on phages or plasmids,
both of which are generally host-specific, although these can often be
25 engineered for other hosts. Other suitable vectors include cosmids and
retroviruses, and any other vehicles, which may or may not be specific for a
given system. Control sequences, such as recognition, promoter, operator,
40 inducer, terminator and other sequences essential and/or useful in the
regulation of expression, will be readily apparent to those skilled in the art.

30 Correct preparation of nucleotide sequences may be confirmed, for
45 example, by the method of Sanger et al. (*Proc. Natl. Acad. Sci. USA*
74:5463-7 (1977)).

50 A DNA fragment encoding an angiogenic inhibitory polypeptide may
35 readily be inserted into a suitable vector. Ideally, the receiving vector has

5 suitable restriction sites for ease of insertion, but blunt-end ligation, for
example, may also be used, although this may lead to uncertainty over
reading frame and direction of insertion. In such an instance, it is a matter
of course to test transformants for expression, 1 in 6 of which should have
10 the correct reading frame. Suitable vectors may be selected as a matter of
course by those skilled in the art according to the expression system
desired.

15 By transforming a suitable organism or, preferably, eukaryotic cell
20 line, such as HeLa, with the plasmid obtained, selecting the transformant
with ampicillin or by other suitable means if required, and adding
tryptophan or other suitable promoter-inducer (such as indoleacrylic acid) if
necessary, the desired polypeptide or protein may be expressed. The extent
of expression may be analyzed by SDS polyacrylamide gel
15 electrophoresis-SDS-PAGE (Lemelli, *Nature* 227:680-685 (1970)).

25 Suitable methods for growing and transforming cultures etc. are
usefully illustrated in, for example, Maniatis (*Molecular Cloning*, A
Laboratory Notebook, Maniatis et al. (eds.), Cold Spring Harbor Labs, N.Y.
30 (1989)).

Cultures useful for production of polypeptides or proteins may
suitably be cultures of any living cells, and may vary from prokaryotic
35 expression systems up to eukaryotic expression systems. One preferred
25 prokaryotic system is that of *E. coli*, owing to its ease of manipulation.
However, it is also possible to use a higher system, such as a mammalian
cell line, for expression of a eukaryotic protein. Currently preferred cell lines
40 for transient expression are the HeLa and Cos cell lines. Other expression
systems include the Chinese Hamster Ovary (CHO) cell line and the
30 baculovirus system.

45 Other expression systems which may be employed include
streptomycetes, for example, and yeasts, such as *Saccharomyces* spp.,
especially *S. cerevisiae*. Any system may be used as desired, generally
50 depending on what is required by the operator. Suitable systems may also

5 be used to amplify the genetic material, but it is generally convenient to use
10 *E. coli* for this purpose when only proliferation of the DNA is required.

10 The polypeptides and proteins may be isolated from the fermentation
5 or cell culture and purified using any of a variety of conventional methods
including: liquid chromatography such as normal or reversed phase, using
HPLC, FPLC and the like; affinity chromatography (such as with inorganic
15 ligands or antibodies); size exclusion chromatography; immobilized metal
chelate chromatography; gel electrophoresis; and the like. One of skill in the
20 art may select the most appropriate isolation and purification techniques
without departing from the scope of this invention.

20 The polypeptides may be generated by any of several chemical
techniques. For example, they may be prepared using the solid-phase
15 synthetic technique originally described by R. B. Merrifield, "Solid Phase
Peptide Synthesis. I. The Synthesis Of A Tetrapeptide", J. Am. Chem. Soc.,
25 83, pp. 2149-54 (1963), or they may be prepared by synthesis in solution. A
summary of peptide synthesis techniques may be found in E. Gross & H. J.
Meinhofer, 4 The Peptides: Analysis, Synthesis, Biology; Modern Techniques
30 Of Peptide And Amino Acid Analysis, John Wiley & Sons, (1981) and M.
Bodanszky, Principles Of Peptide Synthesis, Springer-Verlag (1984).

35 The functional activity and/or therapeutically effective dose of an
angiogenic inhibitory polypeptide or nucleic acid encoding therefor can be
25 assayed in vitro by various methods. For example, where one is assaying for
the ability of the angiogenic inhibitory polypeptides, fragments, and analogs,
to inhibit or interfere with the proliferation of capillary endothelial cells (EC)
40 in vitro, various bioassays known in the art can be used, including, but not
limited to, radioactive incorporation into nucleic acids, calorimetric assays
30 and cell counting.

45 Inhibition of endothelial cell proliferation may be measured by
calorimetric determination of cellular acid phosphatase activity or electronic
cell counting. These methods provide a quick and sensitive screen for
50 35 determining the number of endothelial cells in culture after treatment with
the connective tissue growth factor protein, derivative, or analog of the

invention, and an angiogenesis stimulating factor such as aFGF. The calorimetric determination of cellular acid phosphatase activity is described by Connolly et al., 1986, *J. Anal. Biochem.* 152: 136-140. According to this method, capillary endothelial cells are treated with angiogenesis stimulating factors, such as aFGF, and a range of potential inhibitor concentrations. These samples are incubated to allow for growth, and then harvested, washed, lysed in a buffer containing a phosphatase substrate, and then incubated a second time. A basic solution is added to stop the reaction and color development is determined at 405 λ . According to Connolly et al., a linear relationship is obtained between acid phosphatase activity and endothelial cell number up to 10,000 cells/sample. Standard curves for acid phosphatase activity are also generated from known cell numbers in order to confirm that the enzyme levels reflect the actual EC numbers. Percent inhibition is determined by comparing the cell number of samples exposed to stimulus with those exposed to both stimulus and inhibitor.

The incorporation of radioactive thymidine by capillary endothelial cells represents another means by which to assay for the inhibition of endothelial cell proliferation by a potential angiogenesis inhibitor. According to this method, a predetermined number of capillary endothelial cells are grown in the presence of 3H-Thymidine stock, an angiogenesis stimulator such as for example, bFGF, and a range of concentrations of the angiogenesis inhibitor to be tested. Following incubation, the cells are harvested and the extent of thymidine incorporation is determined.

The ability of varying concentrations of angiogenic inhibitory polypeptides to interfere with the process of capillary endothelial cell migration in response to an angiogenic stimulus can be assayed using the modified Boyden chamber technique.

Another means by which to assay the functional activity of angiogenic inhibitory polypeptides involves examining the ability of the compounds to inhibit the directed migration of capillary endothelial cells which ultimately results in capillary tube formation. This ability may be assessed for example, using an assay in which capillary endothelial cells plated on

collagen gels are challenged with the inhibitor, and determining whether capillary-like tube structures are formed by the cultured endothelial cells.

Assays for the ability to inhibit angiogenesis *in vivo* include the chick chorioallantoic membrane assay and mouse, rat or rabbit corneal pocket assays. See, Polverini et al., 1991, *Methods Enzymol.* 198: 440-450. According to the corneal pocket assays, a tumor of choice is implanted into the cornea of the test animal in the form of a corneal pocket. The potential angiogenesis inhibitor is applied to the corneal pocket and the corneal pocket is routinely examined for neovascularization.

The therapeutically effective dosage for inhibition of angiogenesis *in vivo*, defined as inhibition of capillary endothelial cell proliferation, migration, and/or blood vessel growth, may be extrapolated from *in vitro* inhibition assays using the compositions of the invention above or in combination with other angiogenesis inhibiting factors. The effective dosage is also dependent on the method and means of delivery. For example, in some applications, as in the treatment of psoriasis or diabetic retinopathy, the inhibitor is delivered in a topical-ophthalmic carrier. In other applications, as in the treatment of solid tumors, the inhibitor is delivered by means of a biodegradable, polymeric implant. The protein can also be modified, for example, by polyethyleneglycol treatment.

Diseases, disorders, or conditions, associated with abnormal angiogenesis or neovascularization, and can be treated with a therapeutic compound of the invention include, but are not limited to retinal neovascularization, tumor growth, hemangioma, solid tumors, leukemia, metastasis, psoriasis, neovascular glaucoma, diabetic retinopathy, arthritis, endometriosis, and retinopathy of prematurity (ROP).

The term "effective amount" refers to an amount of the angiogenic inhibitory polypeptide of the invention sufficient to exhibit a detectable therapeutic effect. The therapeutic effect may include, for example, without limitation, inhibiting the growth of undesired tissue or malignant cells, inhibiting inappropriate angiogenesis (neovascularization), limiting tissue damage caused by chronic inflammation, inhibition of tumor cell growth,

5 and the like. The precise effective amount for a subject will depend upon the
subject's size and health, the nature and severity of the condition to be
treated, and the like. Thus, it is not possible to specify an exact effective
amount in advance. However, the effective amount for a given situation can
10 be determined by routine experimentation based on the information
provided herein.

15 The term "pharmaceutically acceptable" refers to compounds and
compositions which may be administered to mammals without undue
toxicity. Exemplary pharmaceutically acceptable salts include mineral acid
salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the
like; and the salts of organic acids such as acetates, propionates, malonates,
20 benzoates, and the like.

25 The angiogenic inhibitory polypeptides of the invention are
administered orally, topically, or by parenteral means, including
subcutaneous and intramuscular injection, implantation of sustained
release depots, intravenous injection, intranasal administration, and the
like. Accordingly, angiogenic inhibitory polypeptides of the invention are
30 preferably administered as a pharmaceutical composition comprising an
angiogenic inhibitory polypeptide of the invention in combination with a
pharmaceutically acceptable carrier. Such compositions may be aqueous
solutions, emulsions, creams, ointments, suspensions, gels, liposomal
suspensions, and the like. Suitable carriers (excipients) include water,
35 saline, Ringer's solution, dextrose solution, and solutions of ethanol,
glucose, sucrose, dextran, mannose, mannitol, sorbitol, polyethylene glycol
(PEG), phosphate, acetate, gelatin, collagen, Carbopol Registered TM ,
vegetable oils, and the like. One may additionally include suitable
preservatives, stabilizers, antioxidants, antimicrobials, and buffering agents,
40 for example, BHA, BHT, citric acid, ascorbic acid, tetracycline, and the like.
Cream or ointment bases useful in formulation include lanolin, Silvadene
Registered TM (Marion), Aquaphor Registered TM (Duke Laboratories), and
the like. Other topical formulations include aerosols, bandages, and other
wound dressings. Alternatively one may incorporate or encapsulate the
45 therapeutic compound of the invention in a suitable polymer matrix or
membrane, thus providing a sustained-release delivery device suitable for
50

5 implantation near the site to be treated locally. Other devices include
indwelling catheters and devices such as the Alzet Registered TM minipump.
Ophthalmic preparations may be formulated using commercially available
10 vehicles such as Sorbi-care Registered TM (Allergan), Neodecadron
5 Registered TM (Merck, Sharp & Dohme), Lacrilube Registered TM, and the
like, or may employ topical preparations such as that described in U.S. Pat.
No. 5,124,155, incorporated herein by reference. Further, one may provide a
15 therapeutic compound of the invention in solid form, especially as a
lyophilized powder. Lyophilized formulations typically contain stabilizing
10 and bulking agents, for example human serum albumin, sucrose, mannitol,
and the like. A thorough discussion of pharmaceutically acceptable
excipients is available in Remington's Pharmaceutical Sciences (Mack Pub.
20 Co.).

15 Nucleic acid (DNA) encoding an angiogenic inhibitory polypeptide of
the invention can be delivered to a host by any method known to those of
25 skill in the art. For example, catheters, injection, intravenous, parenteral,
intraperitoneal and subcutaneous injection, oral or other known routes of
administration. The nucleic acid may be delivered "naked" or via a viral
30 vector or liposome.

The amount of angiogenic inhibitory polypeptide of the invention
35 required to treat any particular disorder will of course vary depending upon
the nature and severity of the disorder, the age and condition of the subject,
25 and other factors readily determined by one of ordinary skill in the art.

The invention also provides a pharmaceutical pack or kit comprising
40 one or more containers filled with one or more of the ingredients of the
pharmaceutical compositions of the invention. Optionally associated with
30 such container(s) can be a notice in the form prescribed by a governmental
agency regulating the manufacture, use or sale of pharmaceuticals or
45 biological products, which notice reflects approval by the agency of
manufacture, use or sale for human administration.

50 The references cited throughout this application are herein
incorporated by reference.

5 The present invention is further illustrated by the following
Examples. These Examples are provided to aid in the understanding of the
invention and are not construed as a limitation thereof.

10 5 EXAMPLES

Example 1:

15 10 Cloning of A1 cDNA from Bovine Aortic Endothelial Cells and Production of Recombinant A1 Protein in Mammalian Cells

A1 cDNA was cloned from bovine aortic endothelial cells by
differential screening. A cDNA phage library was prepared from bovine
aortic endothelial cells by inserting cDNAs into the EcorI and Xho I sites of
pBluscript vector (Sratagene). Approximately 50,000 plaques were lifted
15 with replica filters for differential hybridization. Probes were prepared by
reverse transcription. First, mRNAs were isolated from confluent bovine
aortic endothelial (BAE) cells treated with or without 1 nM tumor necrosis
factor alpha (TNF- α) for 6 hours. Then one microgram of each mRNA was
25 used to synthesize radioactive cDNA probes of up to 2×10^9 cpm/ μ g specific-
activity, using M-MuLV reverse transcriptase. Plaques preferentially
30 hybridized with cDNA probe from untreated BAE cells were cloned and
further analyzed.

35 25 The A1 gene was highly expressed in untreated BAE cells but
dramatically down-regulated in TNF- α treated BAE cells. This warranted
further studies to look at its antiangiogenic activities.

40 The cDNA encoding A1 including the signal peptide was PCR
amplified using Taq DNA polymerase. The sequences of the primers were:

30 5'CTCGAGATGTCAGCCACCGGCCTGGGC3' (SEQ ID NO:4)

45 5'AAGCTTGCCATGTCTCCATACATCTT3' (SEQ ID NO:5)

50 35 The amplified fragment was then inserted into the Xho I and Hind III
sites of pcDNA3.1a, a mammalian expression vector that carries a c-myc
epitope for detection and a polyhistidine sequence for purification of the
resulted fusion protein (Invitrogen). The sequence of the construct was

5 confirmed by automatic sequencing. The construct was transfected into
293T cells for transient expression and CHO cells for stable expression of A1
protein. Recombinant A1 protein was isolated from the conditioned media
10 of the above transfected cells by affinity chromatography using HisBind
5 Resin from Novagen.

Example 2:

Recombinant A1 protein inhibits endothelial cell proliferation *in vitro*.

15
20 The ability of A1 protein to inhibit growth of endothelial cells was
tested by BCE proliferation assay (Folkman et al., *Natl. Aca. Sci. Proc. USA*
76: 5217-5221, 1979). Briefly, cultured bovine capillary endothelial cells
dispersed with 0.05% trypsin/0.53 mM EDTA were plated onto gelatinized
(Difco) 24-well culture plates (12,500 cell/well) in DMEM containing 10%
15 bovine calf serum (BCS) and incubated for 24 hours. The media was
replaced with 0.25 ml DMEM containing 5% bovine calf serum and either
25 buffer only or buffer containing 25 ng/ml to 1 µg/ml of recombinant A1
protein were added. After 20 minutes of incubation, same media containing
bFGF were added to obtain a final volume of 0.5 ml and 1 ng/ml bFGF.
30 After 72 hours, the cells were counted with a Coulter Counter.

35 The recombinant A1 protein potently inhibited endothelial cell
proliferation. At concentration of 1 µg/ml, the A1 protein inhibited bFGF
driven endothelial cell proliferation by 80%. The IC₅₀ (the concentration for
25 half-maximum inhibition) was approximately 50-100 ng/ml. The inhibition
was dose dependent and saturable.

40 Recombinant A1 protein did not inhibit the growth of bovine
fibroblasts in culture, indicating that the effect was endothelial cell specific.

30
45 Example 3:

A1 protein inhibits tumor angiogenesis and tumor growth *in vivo*

50 The same construct used for producing recombinant A1 protein *in*
35 *vitro* was transfected into a human melanoma cell line in culture. Tumor
cells that produced recombinant A1 protein were selected and cloned. To

5 test the effect of A1 protein on tumor angiogenesis and tumor growth *in vivo*,
1x10⁶ human melanoma cells expressing A1 protein or transfected with
vector alone were inoculated subcutaneously into the upper-dorsal region of
10 6-week old male nude mice.

15 A1 over-expression suppressed the growth of human melanoma by
more than 99.9% in nude mice, compared with the tumors transfected with
the vector alone (control tumors). From the end of the second week after
inoculation, A1 transfected tumors grew to 3-6 mm in diameter and then
20 were held at a static stage, while control tumors continued growing to up to
more than ten grams killing the mice. As of the filing date of the present
application, A1 transfected tumors were held static for 5 months in one
experiment and 6 weeks in another (the experiment was still in progress at
the time this application was filed). There was no sign of toxicity. Mice with
25 A1 transfected tumors were healthy and gained weight normally. The long-
term inhibition of tumor growth also indicated that there was lack of drug
resistance.

30 Autopsy and histology studies showed that the A1 transfected tumors
were disc-shaped pigmented tumors composed of a thin layer of viable
tumor cells and a necrotic center. The thickness of viable tumor cell layer
was 100-120 μ throughout the entire tumor, which is exactly the effective
oxygen diffusion distance. This suggests that the cells beyond this distance
35 could not survive because of the lack of neovascularization in the tumor. In
the viable tumor regions, mitotic figures were common but micro vessels
were not found. These are typical histological characteristics of tumors
suppressed by the inhibition of angiogenesis. Therefore, over expression of
40 A1 protein by tumor cells prevented tumor angiogenesis and consequently,
tumor growth *in vivo*.

45 Over expression of A1 protein in human melanoma cells did not alter
cell growth *in vitro*, as compared with the cells transfected with the vector
alone. This indicated that the above antitumor effect was not due to an
anti-growth effect of A1 protein directly against tumor cells.

50 35
Example 4

Functional Domains

Analysis of the A1 peptide sequence revealed 4 functional domains.

These are:

Amino acid	34-100:	IGF (insulin-like growth factor) binding domain The consensus sequence is GCGCCxxC.
	103-166:	vWFC (von Willebrand factor type C repeat) This domain covers cysteines 13-22.
	201-242:	TSP-1 (Thrombospondin type 1 domain) The consensus sequence is WSxCScCG.
	256-330:	CTCK-2 C-terminal cysteine knot profile

The invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements within the spirit and scope of the invention.

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What is claimed is:

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1. A pharmaceutical composition comprising an amount of a peptide that is effective to inhibit angiogenesis, in which the peptide:

- a. has at least one of the following domains: insulin-like growth factor binding domain (consensus sequence: GCGCCxxC), Willebrand factor type C repeat, Thrombospondin type 1 domain (consensus sequence: WSxCSccCG) and C-terminal cysteine knot profile;
- b. inhibits bFGF-stimulated bovine endothelial cell proliferation using a known assay; and
- c. has greater than 80% homology with the corresponding segment of a protein selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3; and a pharmaceutically acceptable carrier.

2. The composition of claim 1, wherein the peptide has at least an insulin-like growth factor binding domain and a von Willebrand factor type C repeat domain.

3. The composition of claim 1, wherein the peptide has at least an insulin-like growth factor binding domain, a von Willebrand factor type C repeat domain and a thrombospondin type 1 domain.

4. The composition of claim 1, wherein the peptide has an insulin-like growth factor binding domain, a von Willebrand factor type C repeat domain, a thrombospondin type 1 domain and a terminal cysteine knot profile.

5. The composition of claim 4, wherein the peptide is represented by SEQ ID NO: 1.

6. The composition of claim 4, wherein the peptide is represented by SEQ ID NO: 2.

7. The composition of claim 4, wherein the peptide is represented by SEQ ID NO: 3.

5

8. The composition of claim 1, wherein the carrier is acceptable for topical application to the skin.

10

9. A pharmaceutical composition comprising a nucleic acid encoding a peptide that is effective to inhibit angiogenesis, in which the peptide:

15

a. has at least one of the following domains: insulin-like growth factor binding domain (consensus sequence: GCGCCxxC), Willebrand factor type C repeat, Thrombospondin type 1 domain (consensus sequence: WSxCScCG) and C-terminal cysteine knot profile;

20

b. inhibits bFGF-stimulated bovine endothelial cell proliferation using a known assay; and

25

c. has greater than 80% homology with the corresponding segment of a protein selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3; and a pharmaceutically acceptable carrier.

30

10. A method of inhibiting atopic angiogenesis in a subject, having a disease or disorder causing atopic angiogenesis requiring such inhibition, which comprises administering to the subject of pharmaceutical composition of claim 1 or 9.

35

11. The method of claim 10, in which the disease or disorder is a solid tumor.

40

12. The method of claim 11, in which the tumor is a tumor of the central nervous system.

45

13. The method according to claim 10, in which the disease or disorder is an ophthalmologic disease or disorder.

50

55

SEQUENCE LISTING

- (1) GENERAL INFORMATION
- (i) APPLICANT: CHILDREN'S MEDICAL CENTER CORPORATION
- (ii) TITLE OF THE INVENTION: METHODS AND COMPOSITIONS FOR INHIBITION OF ANGIOGENESIS
- (iii) NUMBER OF SEQUENCES: 5
- (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: Peabody & Brown
 - (B) STREET: 101 Federal Street
 - (C) CITY: Boston
 - (D) STATE: MA
 - (E) COUNTRY: USA
 - (F) ZIP: 02110
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Diskette
 - (B) COMPUTER: IBM Compatible
 - (C) OPERATING SYSTEM: DOS
 - (D) SOFTWARE: FastSEQ for Windows Version 2.0
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: 09/119,804
 - (B) FILING DATE: 21 July 1998
- (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Resnick, David S
 - (B) REGISTRATION NUMBER: 34,235
 - (C) REFERENCE/DOCKET NUMBER: 40217-PCT
- (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: 617-345-6057
 - (B) TELEFAX: 617-345-1300

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 349 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

Met Ser Ala Thr Gly Leu Gly Pro Val Arg Cys Ala Phe Val Leu Leu
 1          5          10          15
Leu Ala Leu Cys Ser Arg Pro Ala Ser Ser Gln Asp Cys Cys Ser Ala
 20          25          30
Pro Cys Gln Cys Pro Ala Gly Pro Ala Pro Arg Cys Pro Ala Gly Val
 35          40          45
Ser Leu Val Leu Asp Gly Cys Gly Cys Cys Val Cys Ala Lys Gln Leu
 50          55          60
Ser Glu Leu Cys Thr Glu Arg Asp Pro Cys Asp Pro His Lys Gly Leu
 65          70          75          80
Phe Cys Asp Phe Gly Ser Pro Thr Asn Arg Lys Ile Gly Val Cys Thr
 85          90          95
Ala Lys Asp Gly Ala Pro Tyr Ile Phe Gly Gly Thr Val Tyr Gln Ser
100          105          110
Gly Glu Ser Phe Gln Ser Ser Cys Lys Tyr Gln Cys Thr Cys Leu Asp
115          120          125
Gly Ser Val Gly Cys Val Pro Leu Cys Ser Val Asp Val Arg Leu Pro
130          135          140
Ser Pro Asp Cys Pro Phe Pro Arg Arg Val Lys Leu Pro Gly Lys Cys
145          150          155          160
Cys Glu Glu Trp Val Ser Arg Asp Glu Lys Glu His Thr Val Val Gly
165          170          175
Pro Ala Leu Ala Ala Tyr Arg Leu Glu Asp Thr Phe Gly Pro Asp Pro
180          185          190
Thr Met Ile Arg Ala Asn Cys Gln Val Gln Thr Thr Glu Trp Ser Ala
195          200          205
Tyr Ser Lys Thr Cys Gly Met Gly Ile Ser Thr Arg Val Thr Asn Asp
210          215          220
Asn Ala Phe Cys Arg Leu Glu Lys Gln Ser Arg Leu Cys Met Val Arg
225          230          235          240
Pro Cys Glu Ala Asp Leu Glu Glu Asn Ile Lys Lys Gly Lys Lys Cys
245          250          255
Ile Arg Thr Pro Lys Ile Ser Lys Pro Ile Lys Phe Gln Leu Ser Gly
260          265          270
Cys Thr Ser Met Lys Thr Tyr Arg Ala Lys Phe Phe Gly Val Cys Thr
275          280          285
Asp Gly Arg Cys Cys Thr Pro His Arg Thr Thr Thr Leu Pro Val Glu
290          295          300
Phe Lys Cys Pro Asp Gly Glu Val Met Lys Lys Ser Met Met Phe Ile
305          310          315          320
Lys Thr Cys Ala Cys His Tyr Asn Cys Pro Gly Asp Asn Asp Ile Phe
325          330          335
Glu Ser Leu Tyr Tyr Arg Lys Met Tyr Gly Asp Met Ala
340          345

```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 349 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Thr Ala Ala Ser Met Gly Pro Val Arg Val Ala Phe Val Val Leu
1      5      10
Leu Ala Leu Cys Ser Arg Pro Ala Val Gly Gln Asn Cys Ser Gly Pro
20     25     30
Cys Arg Cys Pro Asp Glu Pro Ala Pro Arg Cys Pro Ala Gly Val Ser
35     40     45
Leu Val Leu Asp Gly Cys Gly Cys Cys Arg Val Cys Ala Lys Gln Leu
50     55     60
Gly Glu Leu Cys Thr Glu Arg Asp Pro Cys Asp Pro His Lys Gly Leu
65     70     75
Phe Cys Asp Phe Gly Ser Pro Ala Asn Arg Lys Ile Gly Val Cys Thr
85     90     95
Ala Lys Asp Gly Ala Pro Cys Ile Phe Gly Gly Thr Val Tyr Arg Ser
100    105    110
Gly Glu Ser Phe Gln Ser Ser Cys Lys Tyr Gln Cys Thr Cys Leu Asp
115    120    125
Gly Ala Val Gly Cys Met Pro Leu Cys Ser Met Asp Val Arg Leu Pro
130    135    140
Ser Pro Asp Cys Pro Phe Pro Arg Arg Val Lys Leu Pro Gly Lys Cys
145    150    155
Cys Glu Glu Trp Val Cys Asp Glu Pro Lys Asp Gln Thr Val Val Gly
165    170    175
Pro Ala Leu Ala Tyr Arg Leu Glu Asp Thr Phe Gly Pro Asp Pro
180    185    190
Thr Met Ile Arg Ala Asn Cys Leu Val Gln Thr Thr Glu Trp Ser Ala
195    200    205
Cys Ser Lys Thr Cys Gly Met Gly Ile Ser Thr Arg Val Thr Asn Asp
210    215    220
Asn Ala Ser Cys Arg Leu Glu Lys Gln Ser Arg Leu Cys Met Val Arg
225    230    235
Pro Cys Glu Ala Asp Leu Glu Glu Asn Ile Lys Lys Gly Lys Lys Cys
245    250    255
Ile Arg Thr Pro Lys Ile Ser Lys Pro Ile Lys Phe Glu Leu Ser Gly
260    265    270
Cys Thr Ser Met Lys Thr Tyr Arg Ala Lys Phe Cys Gly Val Cys Thr
275    280    285
Asp Gly Arg Cys Cys Thr Pro His Arg Thr Thr Thr Leu Pro Val Glu
290    295    300
Phe Lys Cys Pro Asp Gly Glu Val Met Lys Lys Asn Met Met Phe Ile
305    310    315    320

```

Lys Thr Cys Ala Cys His Tyr Asn Cys Pro Gly Asp Asn Asp Ile Phe
 325 330 335
 Glu Ser Leu Tyr Tyr Arg Lys Met Tyr Gly Asp Met Ala
 340 345

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 348 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Leu Ala Ser Val Ala Gly Pro Ile Ser Leu Ala Leu Val Leu Leu
 1 5 10 15
 Ala Leu Cys Thr Arg Pro Ala Thr Gly Gln Asp Cys Ser Ala Gln Cys
 20 25 30
 Gln Cys Ala Ala Glu Ala Ala Pro His Cys Pro Ala Gly Val Ser Leu
 35 40 45
 Val Leu Asp Gly Cys Gly Cys Cys Arg Val Cys Ala Lys Gln Leu Gly
 50 55 60
 Glu Leu Cys Thr Glu Arg Asp Pro Cys Asp Pro His Lys Gly Leu Phe
 65 70 75 80
 Cys Asp Phe Gly Ser Pro Ala Asn Arg Lys Ile Gly Val Cys Thr Ala
 85 90 95
 Lys Asp Gly Ala Pro Cys Val Phe Gly Gly Ser Val Tyr Arg Ser Gly
 100 105 110
 Glu Ser Phe Gln Ser Ser Cys Lys Tyr Gln Cys Thr Cys Leu Asp Gly
 115 120 125
 Ala Val Gly Cys Val Pro Leu Cys Ser Met Asp Val Arg Leu Pro Ser
 130 135 140
 Pro Asp Cys Pro Phe Pro Arg Arg Val Lys Leu Pro Gly Lys Cys Cys
 145 150 155 160
 Lys Glu Trp Val Cys Asp Glu Pro Lys Asp Arg Thr Ala Val Gly Pro
 165 170 175
 Ala Leu Ala Ala Tyr Arg Leu Glu Asp Thr Phe Gly Pro Asp Pro Thr
 180 185 190
 Met Met Arg Ala Asn Cys Leu Val Gln Thr Thr Glu Trp Ser Ala Cys
 195 200 205
 Ser Lys Thr Cys Gly Met Gly Ile Ser Thr Arg Val Thr Asn Asp Asn
 210 215 220
 Thr Phe Cys Arg Leu Glu Lys Gln Ser Arg Leu Cys Met Val Arg Pro
 225 230 235 240
 Cys Glu Ala Asp Leu Glu Glu Asn Ile Lys Lys Gly Lys Cys Ile
 245 250 255
 Arg Thr Pro Lys Ile Ala Lys Pro Val Lys Phe Glu Leu Ser Gly Cys
 260 265 270
 Thr Ser Val Lys Thr Tyr Arg Ala Lys Phe Cys Gly Val Cys Thr Asp
 275 280 285

5/5

Gly	Arg	Cys	Cys	Thr	Pro	His	Arg	Thr	Thr	Thr	Leu	Pro	Val	Glu	Phe
290						295					300				
Lys	Cys	Pro	Asp	Gly	Glu	Ile	Met	Lys	Lys	Asn	Met	Met	Phe	Ile	Lys
305				310						315					320
Thr	Cys	Ala	Cys	His	Tyr	Asn	Cys	Pro	Gly	Asp	Asn	Asp	Ile	Phe	Glu
			325						330					335	
Ser	Leu	Tyr	Tyr	Arg	Lys	Met	Tyr	Gly	Asp	Met	Ala				
			340					345							

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 27 nucleic acids
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTC GAG ATG TCA GCC ACC GGC CTG GGC

27

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 27 nucleic acids
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AAC CTT GGC CAT GTC TCC ATA CAT CTT

27

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/13338

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 15/00, 15/63; A61K 38/00, 35/00

US CL : 435/69.1, 320.1; 514/2, 44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 320.1; 514/2, 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, WEST, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database EMBL/GENBANK/DBJ, Accession Number O18739, LILIENSIEK et al., Connective Tissue Growth Factor precursor, 15 July 1998.	1-5, 8-13
X	Database PIR, Accession No. A40551, BRADHAM et al., Connective Tissue Growth Factor - Human. 17 July 1992.	1-4, 6, 8-13
X	Database PIR, Accession No. A53228, RYSECK et al., Fisp-12 Protein Precursor - Mouse, 19 May 1994.	1-4, 7-13
X	Database GENESEQ, Accession No. R79964, BRADHAM et al., Connective Tissue Growth Factor. 12 June 1996.	1-5, 8-13

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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D documents referring to an oral disclosure, use, exhibition or other means

E document published prior to the international filing date but later than the priority date claimed

F later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

A document member of the same patent family

Date of the actual completion of the international search

13 SEPTEMBER 1999

Date of mailing of the international search report

21 OCT 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13338

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BORK, PEER The modular architecture of a new family of growth regulators related to connective tissue growth factor. FEBS Letters. July 1993, Vol. 327, No. 2, pages 125-130, especially page 126, para 2, page 127, para 3, and page 128, para 5.	1-9
X	HASHIMOTO et al. Expression of the Elm1 Gene, a Novel Gene of the CCN (Connective Tissue Growth Factor, Cyr61/Cefg10, and Neuroblastoma Overexpressed Gene) Family, Suppresses In Vivo Tumor Growth and Metastasis of K-1735 Murine Melanoma Cells. Journal of Experimental Medicine. 02 February 1998, Vol. 187, No. 3, pages 289-296, especially abstract and page 294.	1-9, 11, 12
X	US 5,408,040 A (GROTENDORST et al) 18 April 1995, claim i.	1-8
X	US 5,780,263 A (HASTINGS et al) 14 July 1998, column 1, lines 62-62, column 6, lines 33-42, column 11, lines 55-62, and column 12, line 7.	8-13

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- ☐ GRAY SCALE DOCUMENTS
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